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PRINCIPAL INVESTIGATOR: Patrick M. Burke

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, UT 84112

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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Utah
Salt Lake City, UT 84112

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184A1 human mammary epithelial cells (HMECs) form organotypic structures when plated on Matrigel; however, after three weeks on matrigel 184a1 cells do not form polarized, glandular epithelial structures, nor do they polarize if plated onto Transwell tissue culture inserts. Therefore, 184A1s can not be used to address the primary aim of my proposal: to determine if the loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage, enhances motility, or changes the differentiated state of normal cells. HB2 cells are a normal, non-transformed mammary epithelial cell line that expresses high numbers of EGF-R, are tightly adherent to transwells, and polarize on transwells as determined by immunofluorescence localization of ZO-1, the EGF-R and ß-1 integrin. However, HB2 cells do not consistently polarize the EGF-R population. Experiments are underway to determine if conditions can be found in which HB2 reproducibly polarize their EGF-R population. HB2 cells may provide an experimental system with which to address the primary of my proposal. Preliminary experiments comparing 184A1s and HB2 have revealed two interesting and novel findings: 1) rapid internalization of EGF-R can be uncoupled from the normal negative regulatory process of down-regulation; 2) EGF may induce the dephosphorylation of FAK.



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## INTRODUCTION:

### Spatial Distribution of the EGF Receptor System in the Regulation of Breast Epithelial Cell Growth and Organization

The epidermal growth factor receptor (EGF-R) system is necessary for the motility, proliferation and differentiation of human mammary epithelial cells (HMECs) *in vitro*. Additionally, the EGF-R system displays a highly organized spatial distribution *in vivo*. Because the EGF-R system plays a central role in HMEC proliferation and cell motility, it is reasonable to suspect that any defects in its regulation could lead to the clonal expansion of 'premalignant' cell populations. Such expanding clonal populations could give rise to cancerous clones and ultimately metastatic disease. In general, the receptor is subject to two forms of negative regulation, covalent and spatial. Here, I investigate how spatial regulation of the EGF-R influences the physiology of human mammary epithelial cells (HMECs). Epithelial polarization is a form of spatial regulation that effects the function of many molecules including the EGF-R. The spatial distribution of the EGF-R system is highly organized in breast epithelium *in vivo*. *In vivo*, the receptor and one of its ligands, transforming growth factor alpha (TGF– α), are localized to the basolateral surface of mammary alveolar structures; on the other hand, epidermal growth factor (EGF) is synthesized and secreted from the apical side into the lumen of the alveoli. Negative regulation of the EGF-R system could be disrupted by removing the spatial restrictions which segregate one of the ligands from the receptor and/or the receptor from second messenger systems. Down-regulation is another form a spatial regulation where by both surface receptor numbers and total receptor mass are proportionately reduced in response to ligand availability, presumably attenuating signaling by reducing the number of receptors available at the surface (7). Here I investigate the spatial regulation of both the EGF-R and its ligands during HMECs growth and differentiation and determine the consequences of disrupting these regulations.

## SPECIFIC AIMS:

1. Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs.

2. Determine whether a loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage, enhances cell motility, or changes the differentiated state of normally organized epithelial cells.

**STATEMENT OF WORK:**

PART I

Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs. (Months 1-18).

a. Determine the expression levels of the EGF-R and it ligands in normal HMECs. (Months 1-6).
b. Determine the spatial distribution of the EGF-R and it ligands in the organotypic structures of normal HMECs. (Months 7-18).

PART II

Determine whether a loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage to normally organized epithelial cells. (Months 19-36)

a. Disrupt The Polarization Of The EGF-R with two approaches. (Months 19-27).

    1) Overexpression of the wild type receptor.

    2) Transfection of c'958 truncation mutant which localizes to the apical surface of epithelial cells.

b. Assess motility, morphological organization, and growth disrupted cell types. (Months 27-36).

**BODY OF REPORT:**

When my studies began, I proposed working primarily with one cell line, 184A1L5; these cells have been isolated by Martha Stampfer as an immortalized derivative from the parental 184 cell line (1). However, in an effort to be more thorough and in an effort to find a cell line in which I could more closely pursue the original goals of my proposal, I have included the cell line HB2 in many of my investigations. HB2 cells have been isolated by Joyce Taylor-Papadimitriou (3). Both 184A1L5 and HB2 cells are epithelial derivatives, and both express large numbers of EGF-Rs, $3 \times 10^5$ - $7 \times 10^5$, respectively (fig 1). Importantly, the proliferation of 184A1L5 is absolutely dependent upon EGF-R stimulation (fig 2); also, 184A1L5 cells respond motogenicly to EGF-R stimulation (2) and

organize into organotypic structures in the presence of EGF when plated onto the extracellular matrix material Matrigel (fig 3). HB2 cells, on the other hand, do not depend upon EGF-R stimulation for proliferation, will not organize into organotypic structures when plated on matrigel, and are not motile (fig 4). However, to a certain degree, HB2 cells do form polarized epithelium when plated onto tissue culture membranes, and therefore may provide an experimental system in which to investigate the function of epithelial polarization as an EGF-R spatial regulatory mechanism (fig 5).

Experiments with 184A1L5s have demonstrated that rapid internalization of EGF-R can be uncoupled from the negative regulatory mechanism of down-regulation. It has been shown that independent domains of the EGF-R, separable from the kinase domain, are responsible for ligand induced internalization and lysosomal targeting. In fibroblasts and L cells the kinase domain of an occupied EGF-R "activates" these different domains, alters the spatial distribution of the receptor and subsequently causes receptor degradation or down-regulation. This is a classic example of negative spatial regulation (7). I have demonstrated in 184A1L5 (a subclone of 184A1) that rapid internalization of endogenous EGF-R can be occupancy independent and that ligand occupancy in the 184A1L5 cell line is only necessary for lysosomal targeting. Unoccupied EGF-Rs in HB2s, fibroblasts, and transformed cells are internalized at a rate of only 0.02 $min^{-1}$ (i.e.. 2% of total receptors are internalized/min) (fig 6). 184A1L5 cells internalize unoccupied EGF-Rs at a rate of about 0.20 $min^{-1}$, approximately equivalent to the internalization rate of occupied EGF-R in HB2 cells and transformed cells (0.18 $min^{-1}$) (fig 6) (8). Additional experiments, including inside/surface ratios of unoccupied receptors and immunofluorescence, support the conclusion that empty receptors are being rapidly internalized (fig 7 - 9 ). Also importantly, this endocytic activity appears to be specific to the EGF-R as the endocytosis of the transferrin receptor and the fluid phase are similar in HB2 and 184A1L5 cells (fig 10). Despite the ten-fold more rapid internalization of empty EGF-R in 184A1L5, the half-life of EGF-R in the absence of EGF for 184A1L5 and HB2s is very similar (fig 11). This indicates that the internalized empty receptors in 184A1L5 are being recycled. Occupancy of EGF-R in 184A1L5 cells rapidly targets receptors for degradation (fig 11), additionally, high concentrations of exogenous EGF reduces receptor half-life in both HB2 and 184A1L5 cells similarly (fig 11). These findings indicate that the individual steps of down-regulation can be uncoupled, and that intracellular trafficking of the EGF-R is regulated by signals other than receptor occupancy. Much of these findings were reported in a preliminary form in my 1996 Annual report. Since that time, the findings have been confirmed and all the important controls have been done.

Mathematical models have been developed that describe the process of down-regulation, and the equations allow us to make testable predictions (5). In light of my findings, a collaborating graduate student, Greg Oehrtman, re-analyzed these equations and made the prediction that, if there is a large internal pool of unoccupied EGF-R then the percentage of ligand that is internalized and then

escapes by chance will decrease (fig 12). In other words, if a ligand molecule escapes form an internalized receptor, the chance that it will be recaptured within the cell is greater since there are additional unoccupied receptors present. I tested this prediction.

To test this prediction, I preformed experiments like those published elsewhere. Briefly, cells were loaded up radioactive EGF. This was done in such a way as to allow the cells to come to a steady state equilibrium, or with a short pulse of ligand. Surface bound ligand was removed, and the release of intact EGF and mono-iodotyrosine was followed in the supernatant. The ratio of these to species indicate the fraction that is escaping versus the fraction that is being degraded (EGF:mono-iodotyrosine). Results were quantified by native-PAGE and phosphoranalysis software. The actual results can be found in figure 13. Quantification of the results can be found in figure 14. The results indicate that the predictions was incorrect suggesting that either the mathematical model is incorrect or that it is at least too great of an over simplification.

While performing control experiments, I found that EGF-R stimulation may result in the dephosphorylation of focal adhesion kinase (FAK); I am actively pursuing this observation. As mentioned above, EGF stimulates the motility of 184A1L5 cells. It is well documented that EGF also stimulates the motility of other cells. Although there are no direct findings linking FAK activation state to the production of motility, FAK is a major signalling molecule in focal adhesions and focal adhesions are important sights of adhesion during cell migration. Cell migration is critical step during the progression and development of metastatic disease, and understanding the molecular mechanism of motility may aid in being able to combat the process during metastasis. It is possible that over-expression of the EGF-R increases the motility HMECs, through its effect on FAK, and in this way, contribute to the progression of the cancer. Thus far only indirect immunofluorescence experiments suggest that FAK is dephosphorylated (fig 15). The addition of EGF to HB2 cells results in the decrease of phosphotyrosine found in focal adhesions, and concomitantly a loss of FAK from focal adhesions. A variety of hypothesis can be formulated, but the most straight forward hypothesis, since FAK is tyrosine phosphorylated itself, is that EGF stimulation results in the dephosphorylation and redistribution of FAK. These experiments are being preformed in 184A1L5 cells and by using additional techniques. Stimulation of the insulin receptor has been reported to have a similar effect (6).

In order to address the original aims of my proposal I have been in search of the proper experimental cell system. Last year I reported that HB2 cells may serve as an effective model for studying the functional importance of a polarized distribution of the EGF-R system. Initial immunofluorescence results strongly suggested that HB2 cells polarize when plated onto tissue culture membranes (Costar, Transwells) as determined by the distribution of ZO-1 and Beta-1 integrin (fig 5). However, more recent and more quantitative analysis suggest that HB2 cells do not polarize on transwells in a reproducible way.

Radioactive ligand binding experiments indicate that at time the EGF-R distribution is primarily basolateral, but not always. Various approaches have been tried to more reproducibly induce polarization, however, they have been largely unsuccessful. Studies to achieve polarization are still underway as unpublished observations of a collaborator, in another polarized cell type, indicate that EGF-R signaling patterns generated from apical or basolateral membrane domains are different, suggesting that polarized spatial regulation could be very important form of regulation influencing cell physiology.

Although my SOW indicates that I should have isolated clones of cells overexpressing wild type and mutant EGF-R constructs, this has not yet been accomplished. Work is on going. Traditional forms of gene transfer do not work well in either cell line, so we have been forced to develop retroviral vectors for the purpose of transduction. Constructs have been made and transductions are under way. Results are inconclusive at this point; however, the clones will eventually be isolated, and the effects of EGF-R over expression and mutation will be analyzed.

**CONCLUSIONS:**

1.) Individual steps of receptor down-regulation can be uncoupled, and intracellular trafficking of the EGF-R is regulated by signals other than receptor occupancy.

2.) An large internal pool of unoccupied EGF-R is not sufficient to decrease ligand escape efficiencies.

3.) FAK may be dephosphorylated as the result of EGF-R stimulation.

4.) HB2 cells do not polarize reproducibly with regard to subcellular EGF-R distribution.

5.) 184A1L5 and HB2 cells transfect poorly by classical CaPO4 protocols and lipotransfectant reagents.

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## Appendix

*Figure 1*

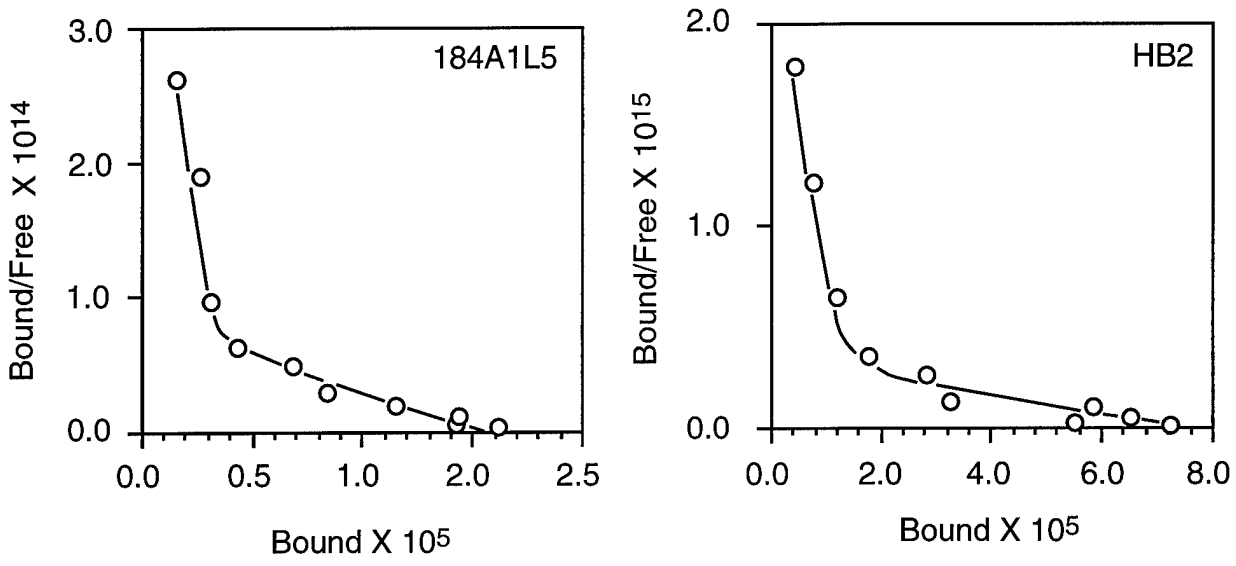



**Figure 1**: Scatchard plots of surface EGF-R using radiolabeled EGF. Non-specific binding was less than 2% of specific counts.

*Figure 2*: Growth of 184A1L5 in Response to EGF and mAb 225

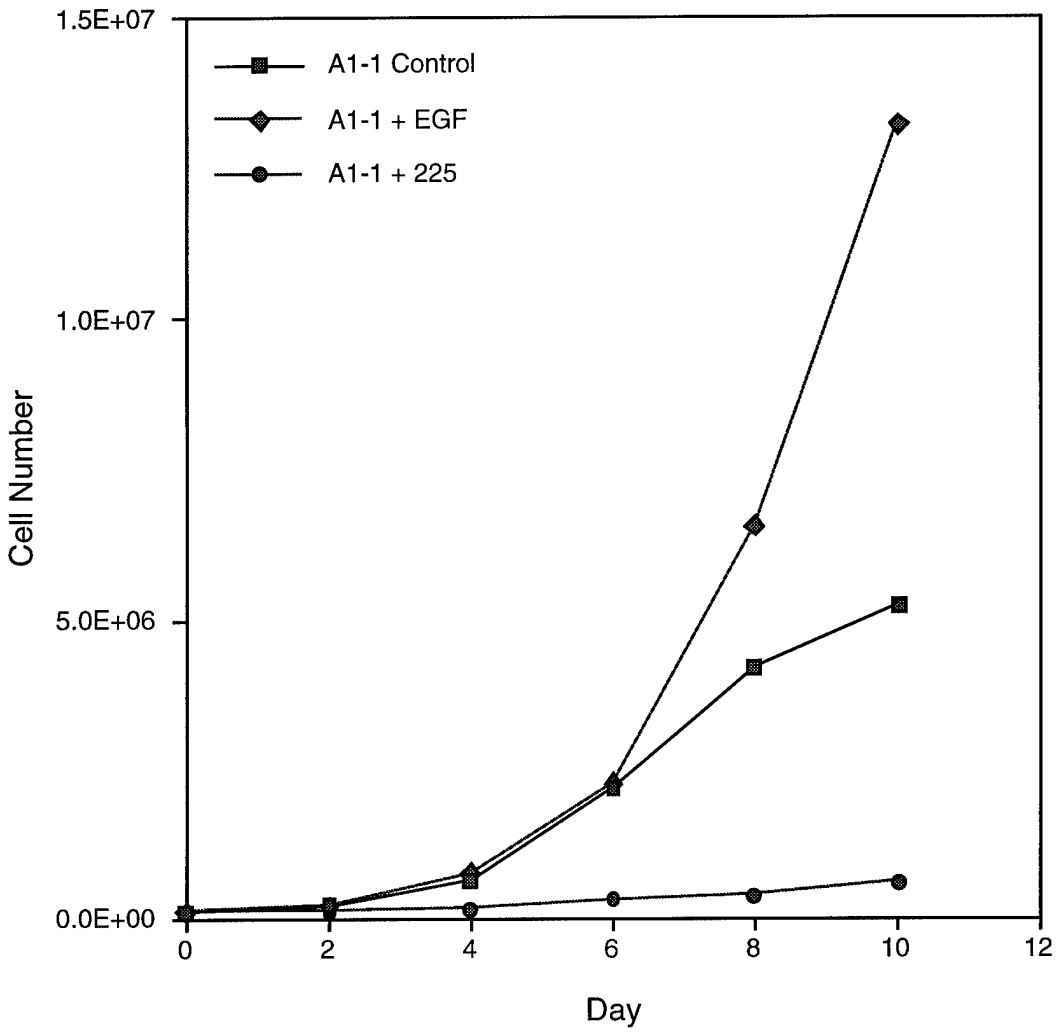



Figure 2: 184A1L5 cells were plated at ~1:300 dilution of a confluent plate. The cells were then grown either in the absence of any additive, in the presence of EGF or in the presence of EGFR antagaonistic antibody 225. Cells were trypsinized and counted every 2 days for 10 days.

Figure 3: 184A1L5 grown on plastic and Matrigel

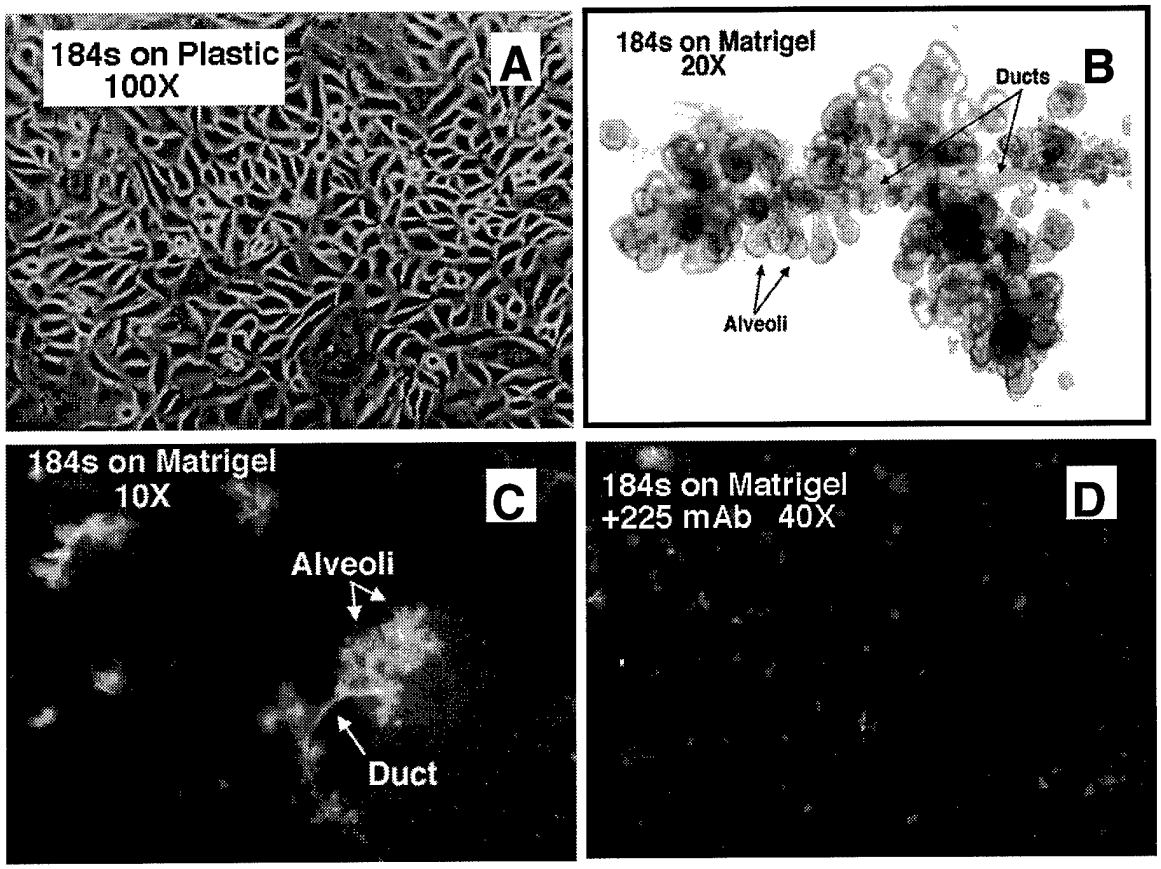


*Figure 4*

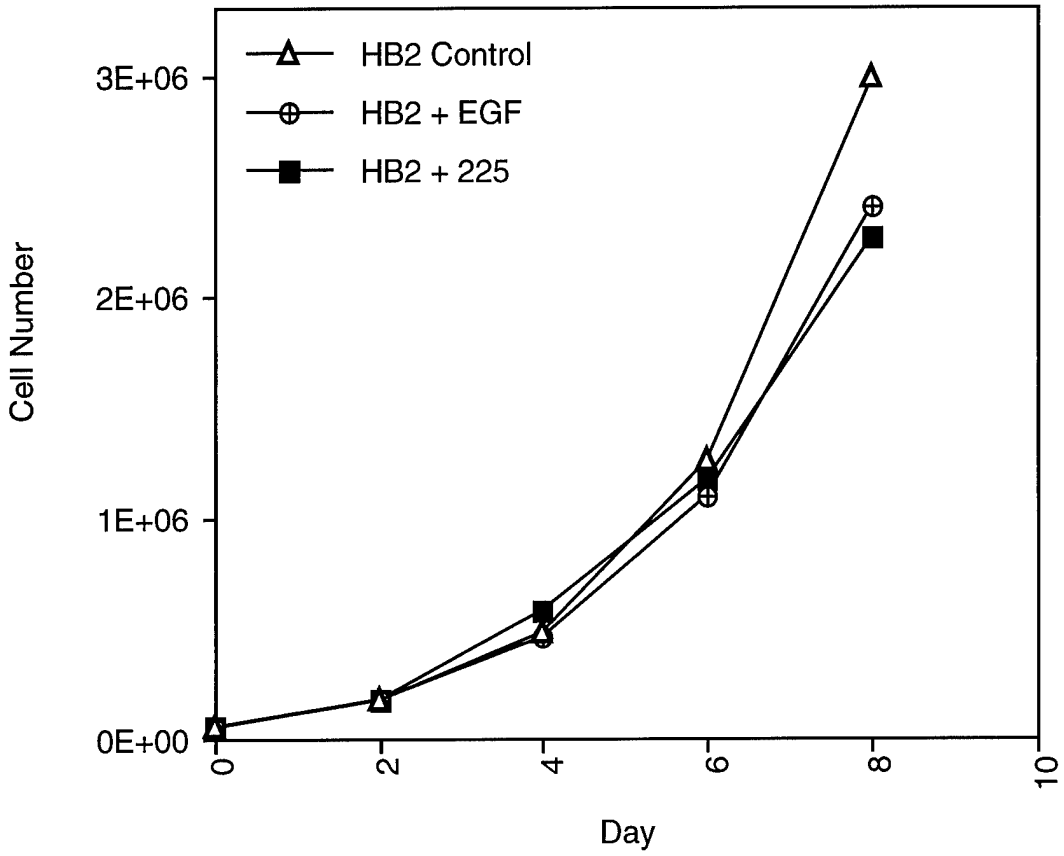
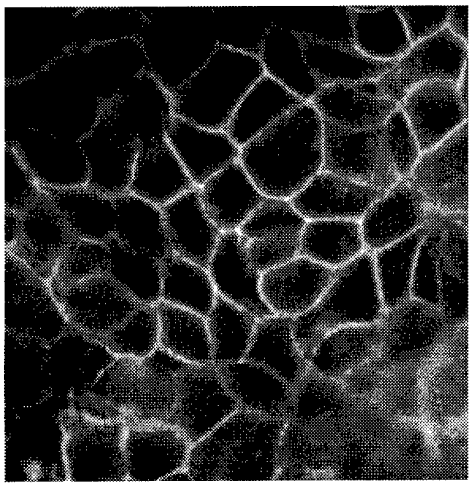


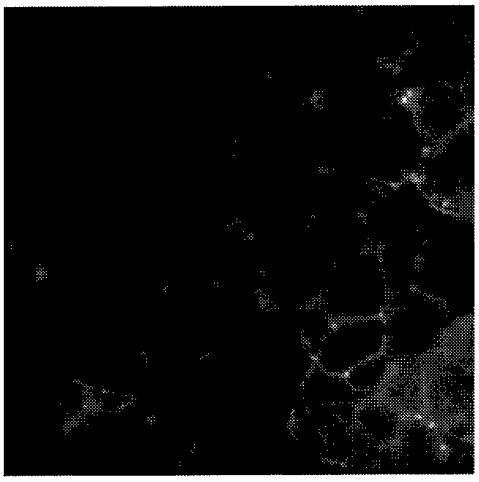

Figure 4: HB2 cell growth in the presence and absence of either EGF, @ 20ng/ml, or EGFR antagonistic antibody 225, @ 20µg/ml. Media was changed every two days and cell number was determined with a coulter counter. Although there appears to be a marginal effect of 225 on HB2 growth, it is not significant; repeat experiments have not been able to reproduce the effect.

## Appendix

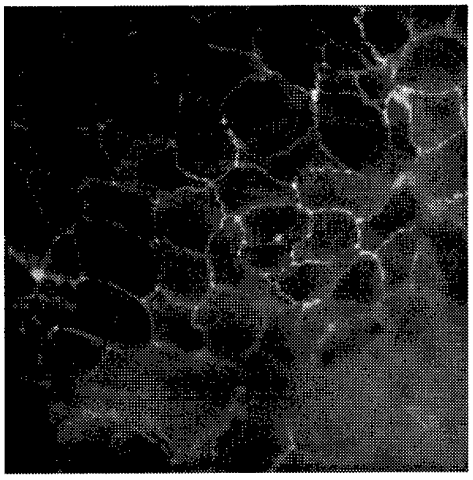
*Figure 5*



ß-1 Integrin



ZO-1 at the ß-1 Integrin Plane



ZO-1 Apical to the ß-1 Integrin Plane

Figure 5: Subcellular localization of ß-1 intergrin and ZO-1 by indirect immunoflourescence in HB2 cells. The ZO-1 marker indicates the apical-basolateral boundry. The ß-1 staining pattern is consistant with basolateral localization.

## Appendix

*Figure 6*

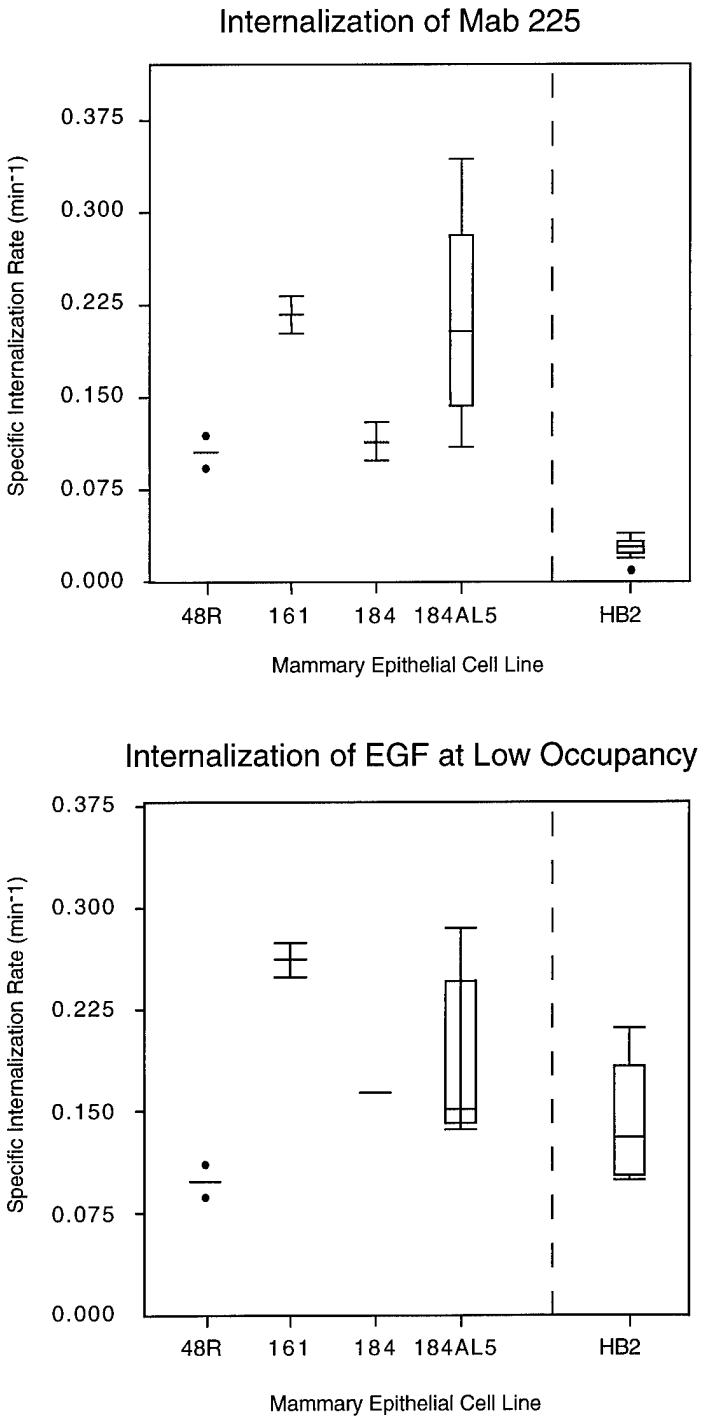



Figure 6: Internalization rate constants of EGF at low concentrations and antagonistic monoclonal antibody 225 were determined in HB2and 184A1L5 cell lines. The same values were also determined for other cell lines, 184, 161 and 48R. These other cell lines are mortal cell lines derived from human mammary epithelia as the 184A1L5 cell line, however they have never been treated with mutagens for the purpose of immortalization.

## Appendix

*Figure 7*

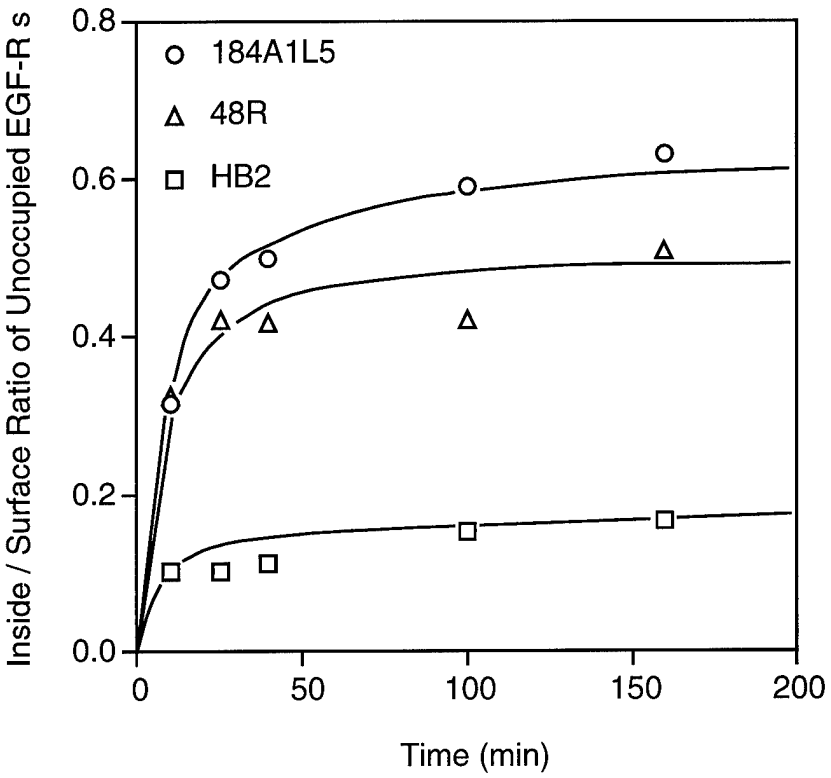



Figure 7: Inside:Surface ratio of unoccupied EGF-R in 184A1L5, HB2 and 48R cells. Cells were incubated in the presence of radiolabeled Mab 225 and the inside and suface associated counts were collected and counted over the time course. These results indicate the steady state distribution of EGF-Rs.

## Appendix

*Figure 8*

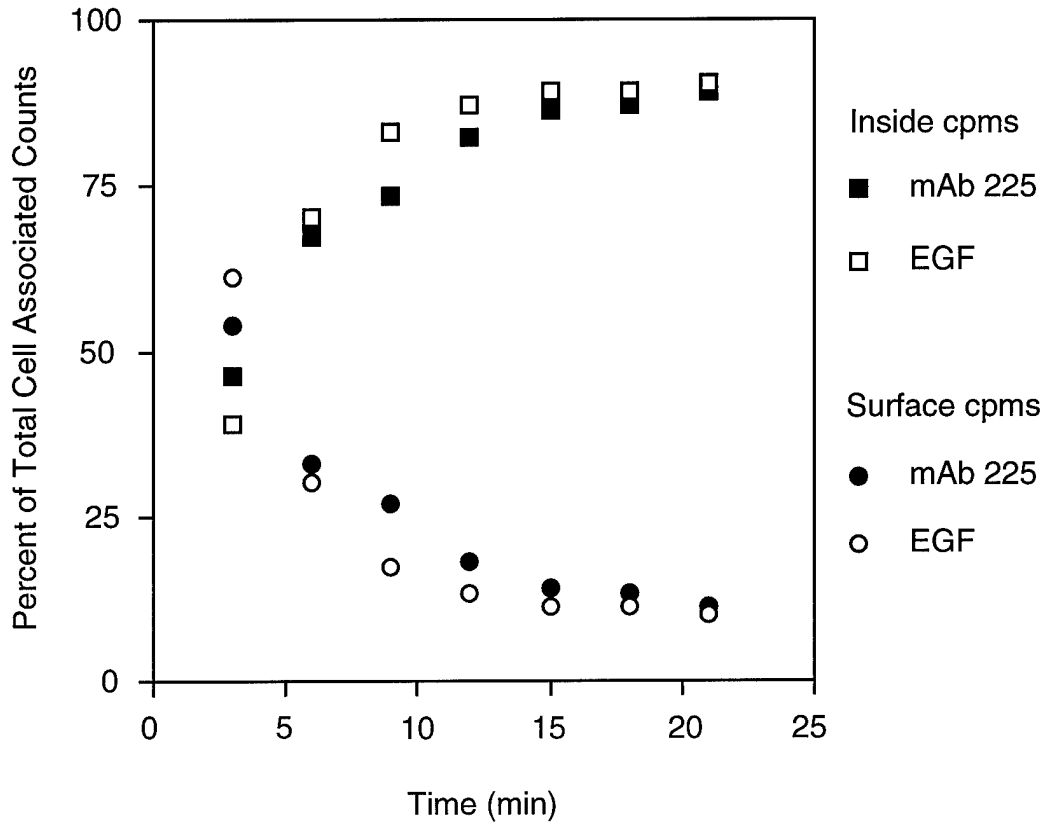



Figure 8: Internalization of EGF and Mab 225 in 184A1L5 cells. Radiolabeled ligands were bound to the cell surface at 4 degrees C for 3 hours. Media was removed, cells were warmed to 37 degrees C and the internalization and clearence from the cell surface of each ligand was followed

## Appendix

*Figure 9*

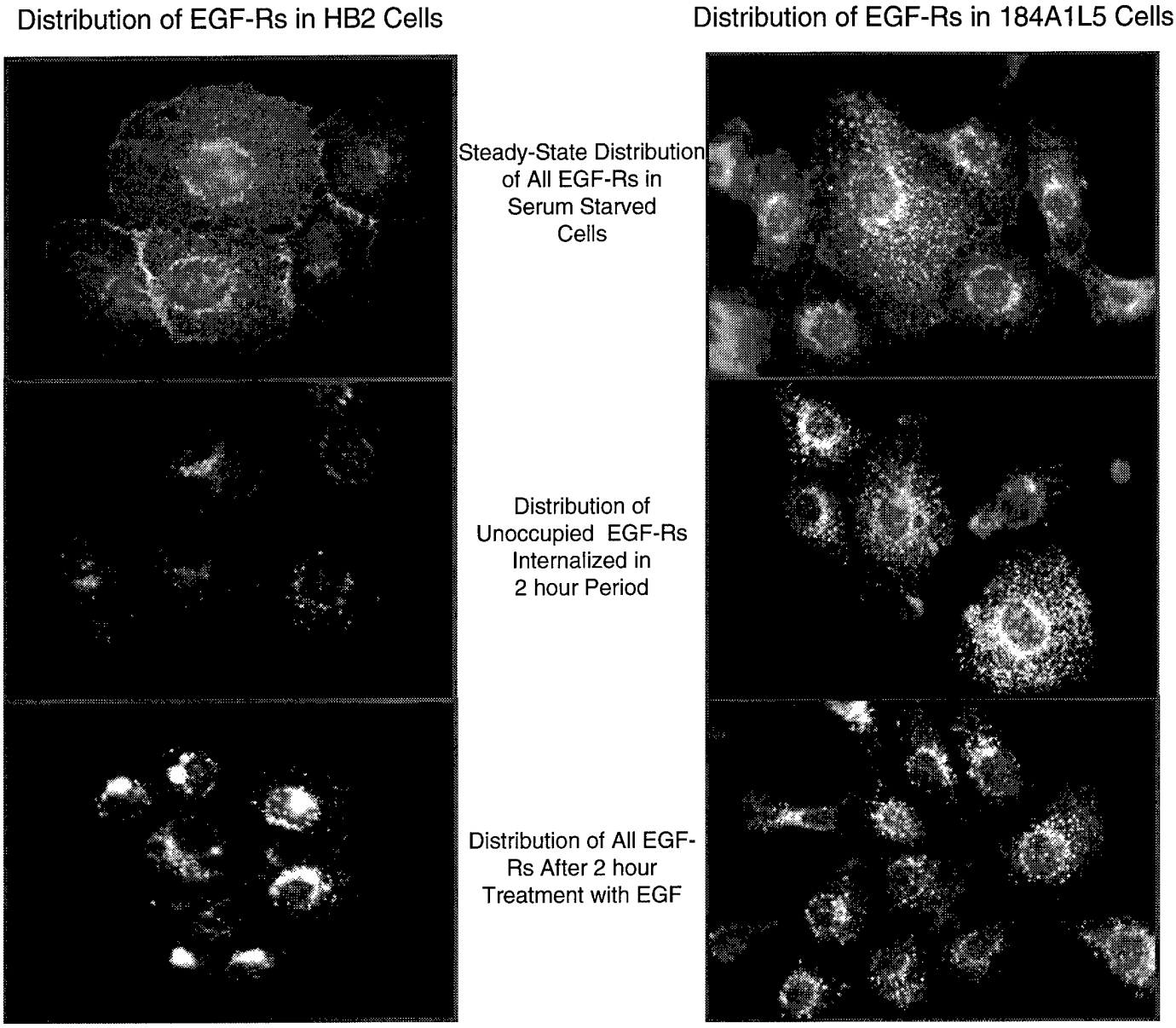



Figure 9: Cells were serum starved for 24 hrs prior treatment with either mAb 225 @ 500ng/ml or EGF @ 50 ng/ml or nothing (control) and kept at 37 degrees centigrade. Control cells and EGF treated cells were fixed and permeablized with 4% paraforaldehyde/ 0.1% Triton X-100. EGF-R was detected using primary antibodies mAb 225 and 13A9 (15 & 10 μg/ml respectively) followed by FITC goat anti-mouse secondary antibody. Cells treated with mAb 225 were handled similarly, but no primary antibodies was used post fixation. Distribution of the mAb 225 "fed" to cells was detected by FITC goat anti-mouse. The control HB2 cells are another non-tumoragenic mammary epithelial cell line of different origin; it was kindly provided by Dr. Joyce Taylor-Papadimitriou.

## Appendix

*Figure 10*

| Cell Line | Fluid Phase Endocytosis (nl/hr * $10^6$ Cells) | Endocytosis of Tf-R (Ke) |
|---|---|---|
| A1-1 | - EGF 143<br>+ EGF 153 | 0.412+/- 0.037 |
| HB2 | - EGF 130<br>+ EGF 218 | 0.435 +/- 0.046 |
| MDA-MB-239 | - EGF 120<br>+ EGF 143 | ND |

Figure 10: Endocytosis controls. Fluid phase endocytosis was determined by measuring the pool of soluable internalized horse radish peroxidase. Ke of the transferrin receptor was determined using standard techniques (the same used to measure that of the EGF-R.

*Figure 11*

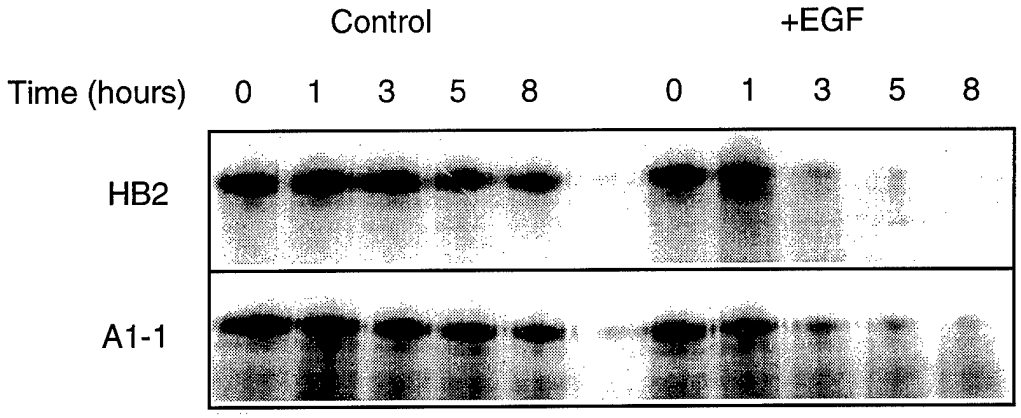



Figure 11: EGF-R trafficking in the absence and presence of EGF in 184A1L5 and HB2 cells. A standard pulse chase experiment was performed. Cells were labeled with radiolabelled with 35S met/cys for 24 hours and then chased for designated time periods with excess unlabeled met/cys. EGF-R was immunoprecipitated with mAb 225, run on a 5-15%gradient gel, exposed to a phosphoimager plate and the cpm /EGF-R band was quantitated.

*Figure 12*

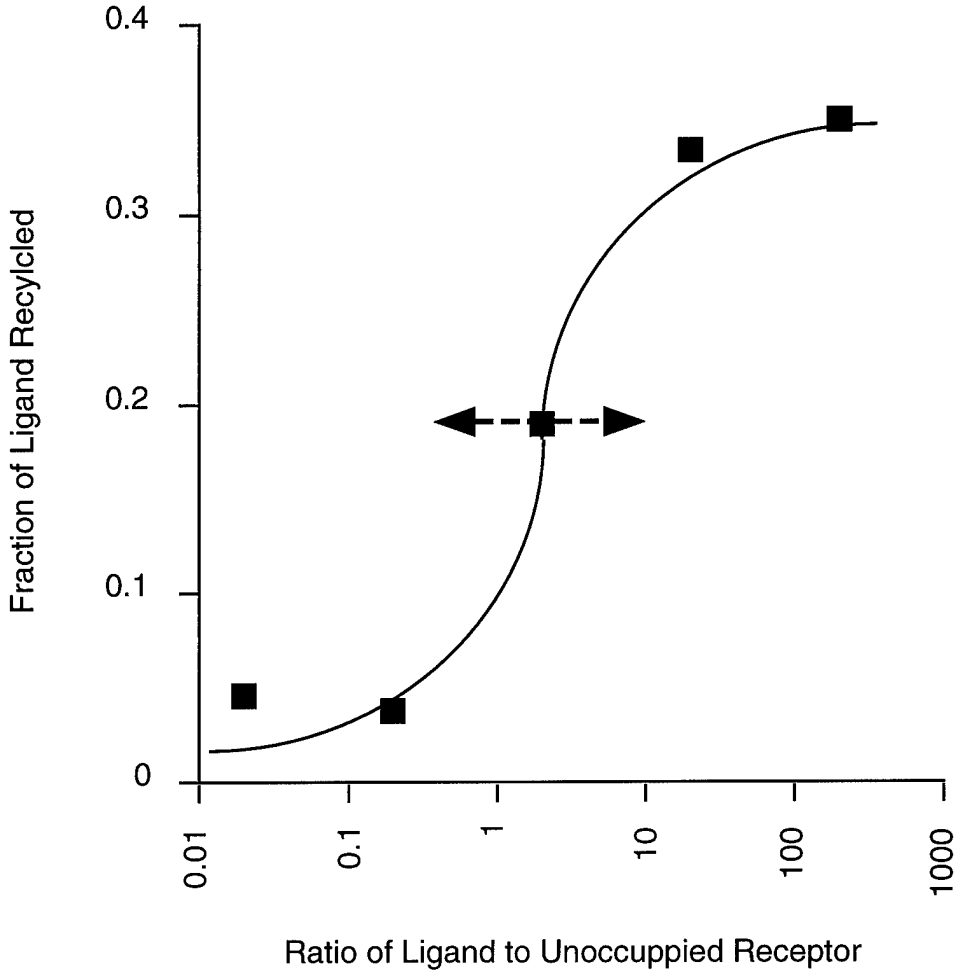



Figure 12: mathematical model describing the relationship the dependence of ligand recylcing on the ratio of iland:internal unoccupied EGF-R

*Figure 13*

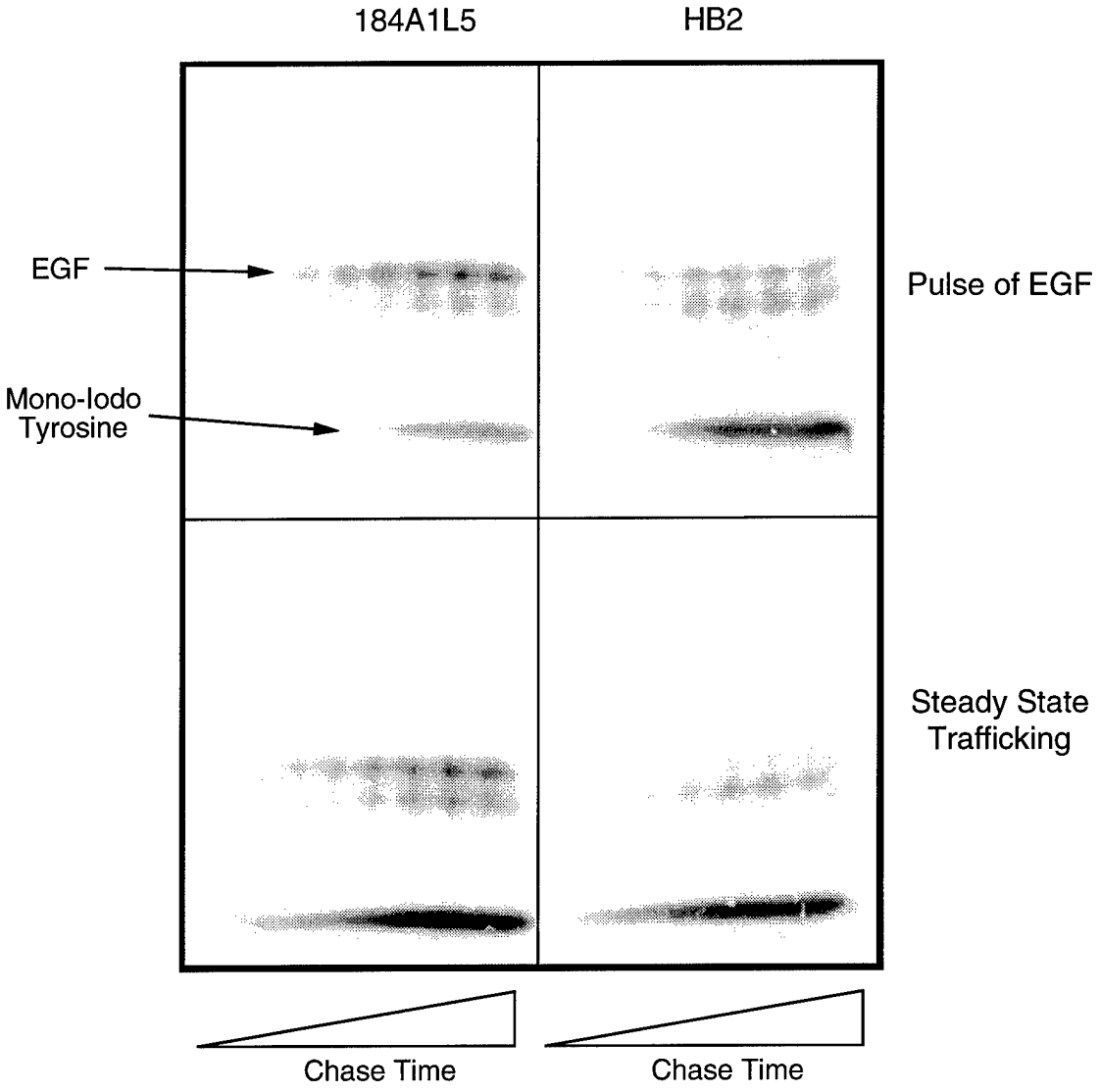



Figure 13: Ligand Trafficking Experiments in HMECs. Cells were loaded with radioactive EGF. This was done in such a way as to allow the cells to come to a steady state equilibrium, or with a short pulse of ligand. Surface bound ligand was removed, and the release of intact EGF and mono-iodotyrosine was followed in the supernatant the the presence of excess unlabeled ligand to prevent to uptake of escaped ligand.

*Figure 14*

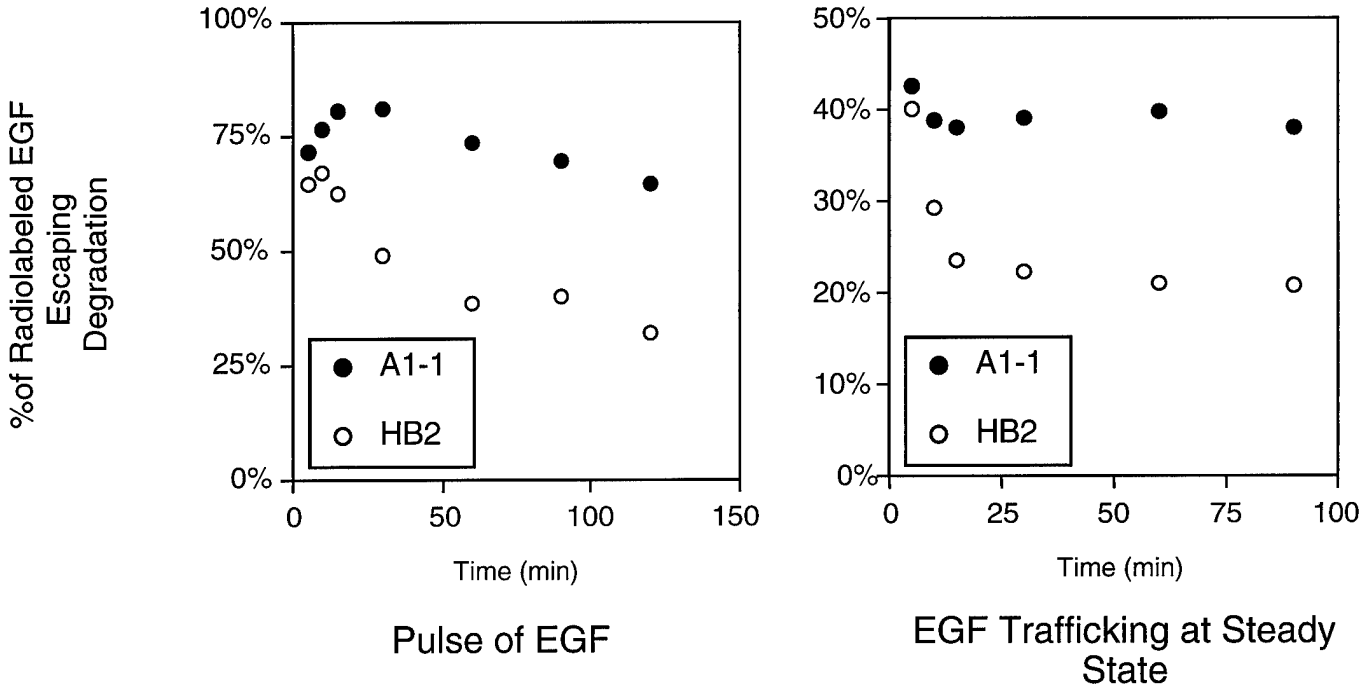



Figure 14: Phosphoranalysis of gels shown in in figure 13

*Figure 15*

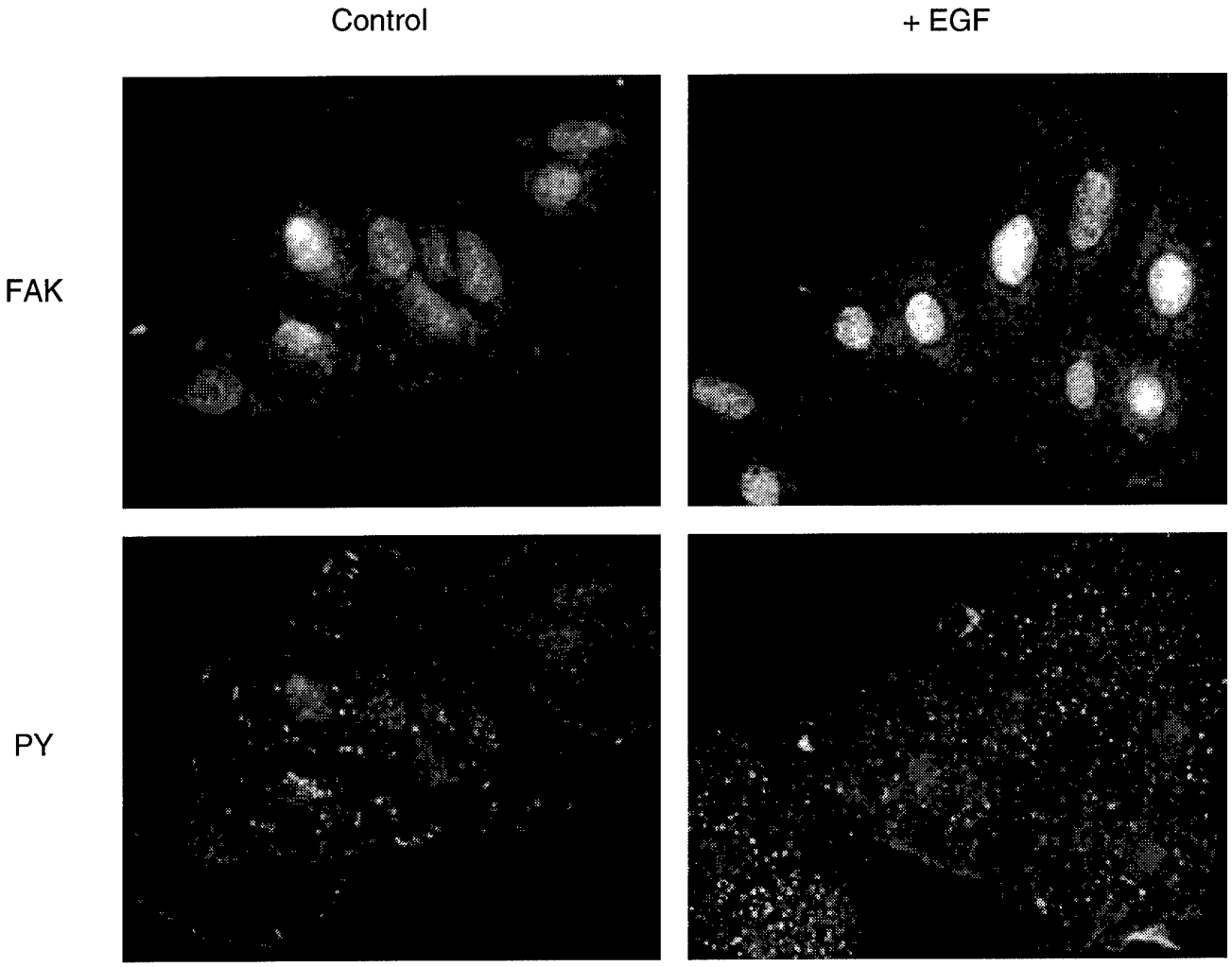



Figure 15: Immunolocalization of FAK and phosphotyrosine in HB2 Cells in the presence and absence of EGF. Cells were plated onto fibronectin coated coverslips and then treated with or with 50 ng/ml EGF for 15 min at 37 degrees C.